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(54) Title: METABOLITES OF GINSENG SAPONINS BY HUMAN INTESTINAL BACTERIA AND ITS PREPARATION FOR AN ANTICANCER			
(57) Abstract			
<p>This invention relates to ginsenoside Me with formula (1), an intestinal flora metabolite of ginseng saponin and anticancer agent containing it as an active ingredient. In addition to a novel compound, the anticancer agent of this invention consists of one active ingredient selected from compound K, compound Y or 20(S)-protopanaxatriol, intestinal flora metabolites of ginseng saponin, together with one or more pharmaceutically acceptable carriers. Said agent is a novel type of potential anticancer agent since it has immunopotentiating actions including inhibitory actions on the vascularization of tumors and extravasation of cancer cells.</p>			
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**Metabolites of ginseng saponins by human intestinal
bacteria and its preparation for an anticancer.**

FIELD OF THE INVENTION

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This invention relates to the metabolites of ginseng saponins by human intestinal bacteria and its preparation for an anticancer and more particularly, to a novel saponin of a metabolite of *Panax ginseng* saponin by human bacteria 10 and a novel preparation of anticancer agent containing a novel saponin of a metabolite of *Panax ginseng* saponin by human bacteria, which exhibits immunopotentiating actions including inhibitory actions on the vascularization of tumors and extravasation of cancer cells.

15

DESCRIPTION OF THE RELATED ART

In recent years much of the development of novel anticancer agents has widely focused on natural sources and 20 synthetic compounds.

Among saponins extracted from *Panax ginseng*, for example, ginsenoside Rh₂ [3-0- β -D-glucopyranosyl-20(s)-

protopanaxadiol] was reported to inhibit the proliferation of liver cancer cells (reference: Japanese Patent No. 89-28759).

Further, both ginsenoside Rg₃ [3-O-[β -D-glucopyranosyl(1 \rightarrow 2)- β -D-glucopyranosyl]-20(R)-protopanaxadiol] and ginsenoside Rb₂[20-O-[α -L-arabinopyranosyl(1 \rightarrow 6)- β -D-glucopyranosyl-3-O-[β -D-glucopyranosyl(1 \rightarrow 2)- β -D-glucopyranosyl]-20(S)-protopanaxadiol] were reported to inhibit the vascularization of tumors and extravasation of cancer cells including inhibitory actions on the metastasis of cancer cells [References: Japanese Patent No. 89-28759, Sato et al.: Biol. Pharm. Bull., 17, 635(1994)].

In case of 20-O-[β -D-glucopyranosyl]-20(S)-protopanaxadiol, called as "compound K" and 20-O-[α -L-arabinopyranosyl(1 \rightarrow 6)- β -D-glucopyranosyl]-20(S)-protopanaxadiol, called as "compound Y", which were isolated from soil strains of Panax ginseng saponin and intestinal flora of rats, their structure was already determined [References: Yoshioka et al.: Chem. Pharm. Bull., 20, 2418(1972), Takino et al.: Medicinal ginseng '69 (Public Publishing Co., Ltd. 267(1989)).

Also, the structure of 20(S)-protopanaxatriol, isolated by sapogenin of Panax ginseng saponin, was already established (Nagai et al.: Tetrahedron, 27, 881(1971)).

The pharmacological actions of these compounds, for

example, inhibition of glucose transplant related to cancer cells by the blockage of membrane protein, were merely reported by each inventor [Hasegawa et al., Planta Med., 60, 197(1994)], including the report on methicillin-resistant 5 bacteria and the inhibition of the excretion of drugs on the multidrugs-resistant cancer cells [Hasegawa et al.: Phytother. Res., 9, 260(1996), Hasegawa et al., Planta Med., 61, 409(1995)].

In case of the conventional chemotherapeutics which exhibit their therapeutic effects by attacking the cancer 10 cells directly, their adverse effects are quite severe. During several decades, any antineoplastic agents with new mode of mechanism have not yet to be on the market. Further, in the event that Panax ginseng saponins are applied for the treatment of some diseases, these substances are reported to 15 be metabolized by intestinal bacteria and said bacteria is liable to be influenced by human's constitution and his food pattern. Thus, any individual differences in the metabolism of saponins may lead to the individual differences in his treatment.

SUMMARY OF THE INVENTION

In view of these situations, the inventors of this invention have investigated the metabolism of ginseng saponin associated by human intestinal bacteria and succeeded in isolating and identifying the following 5 compounds, i.e., a) protopanaxadiol saponins(ginsenoside Rb₁, ginsenoside Rb₂ and ginsenoside Rc), b) compound K, compound Y and 20-O-[α -L- arabinofuranosyl(1 \rightarrow 6)- β -D-glucopyranosyl-20(S)-protopanaxadiol], which are called as ginsenoside Mc, metabolites of ginsenoside Rd, and c) 20(S)-protopanaxatriol, a 10 metabolite of ginsenoside Rg₁ and ginsenoside Re which belongs to protopanaxatriol saponin.

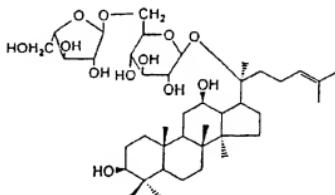
The inventor ascertains that these intestinal flora metabolites are absorbed from intestinal tracts to blood and excreted via urine and feces. By assuring that said 15 intestinal flora metabolites prove to be main substances of Panax ginseng saponin, the inventor has endeavored to develop the therapeutic dosage form containing the active ingredient of saponin, which is not influenced by the difference of intestinal bacteria. As a result of reviewing these 20 physiological activities, the inventor has discovered a novel preparation of anticancer agent , which exhibits immunopotentiating actions including inhibitory actions on

the vascularization of tumors and extravasation of cancer cells. Thus, this invention has finally completed.

Therefore, the object of this invention is to provide a new compound of 20-O-[α -L-arabinofuranosyl(1 \rightarrow 6)- β -D-5 glucopyranosyl-20(S)-protopanaxadiol] having the following formula, a novel ginseng saponin metabolite by human intestinal bacteria(called as ginsenoside Mc) with the following characteristics.

1) Structural formula

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2) Molecular formula: C₄₁H₇₀O₁₂

3) The mass spectrum (Fab-MS, negative and m/z) showed signals at 753[M-H]⁻, 621(M-arabinofuranose-H)⁻, and 459[M-arabinofuranos-glucopyranos-H]⁻.

20 4) The ¹H-NMR spectrum(d5-pyridine) showed signals at δ 3.39(1H, t, J=10.5, 5.1Hz, H-3), 0.80(1H, d, J=11.0Hz, H-5), 3.93(1H, ddd-like, H-12), 5.32(1H, t, J=7.1Hz, H-24), 0.92(3H, s, Me-18), 0.89(3H,

s, Me-19), 1.69(3H, s, Me-21), 1.67(3H, s, Me-26), 1.67(3H, s, Me-27), 1.21(3H, s, Me-28), 1.01(3H, s, Me-29), 0.99(3H, s, Me-30), 5.10(1H, d, J=7.8Hz, H-1'-20-glucopyranosyl), 5.61(1H, J=1.7Hz, H-1''-6'-arabinofuranosyl).

5 5) The ¹³C-NMR spectrum(d5-pyridine) for aglycon moiety showed signals at 839.5(C-1), 28.3(C-2), 78.2(C-3), 39.5(C-4), 56.5(C-5), 18.8(C-6), 35.2(C-7), 40.2(C-8), 50.4(C-9), 37.4(C-10), 30.8(C-11), 70.3(C-12), 49.5(C-13), 51.5(C-14), 30.9(C-15), 26.7(C-16), 51.8(C-17) 16.3(C-18), 16.1(C-19), 83.2(C-20), 22.4(C-21), 36.2(C-22), 23.2(C-23), 10 126.1(C-24), 131.0(C-25), 25.8(C-26), 17.9(C-27), 28.7(C-28), 16.4(C-29), 17.5(C-30);

15 The ¹³C-NMR spectrum(d5-pyridine) for 20-glucopyranosyl moiety showed signals at 98.1(C-1'), 75.1(C-2'), 79.2(C-3'), 72.2(C-4'), 76.5(C-5'), 68.5(C-6');

The ¹³C-NMR spectrum(d5-pyridine) for 6'-arabinofuranosyl moiety showed signals at 110(C-1''), 83.5(C-2''), 79.0(C-3''), 86.3(C-4''), 62.8(C-5'').

20 In addition to ginsenoside compound Mc, a novel intestinal bacteria metabolite of novel ginseng saponin, another object of this invention is to provide an anticancer

agent containing one active ingredient selected from intestinal bacteria metabolites of ginseng saponin such as compound K, compound Y, ginsenoside Mc and protopanaxatriol, together with one or more pharmaceutically acceptable carriers. These intestinal bacteria metabolites of ginseng saponin exhibits remarkable antineoplastic effects in the long run since they potentiate the inhibitory actions against cancer cells in lymphocyte and inhibit the vascularization of tumors and extravasation of cancer cells.

10 Even though the severity of symptoms differs, the oral dose of anticancer agent for adult according to this invention is 1-50mg/60kg of body weight once daily or several times per a day, preferably 3-15mg/day /60kg of body weight.

15 The anticancer agent according to this invention contains either a single ingredient, or said ingredient plus one or more pharmaceutically acceptable carriers such as excipients in the form of solid or liquid.

The administration method and available dosage forms are as follows:

20 a) Oral forms: powders, tablets, suspensions, emulsifiers, capsules, granules, troches, pills, suspensions, spirits, syrups and limonades;

b) Injectable forms, or

c) Topical forms: ointments, solids, suspensions, powders, paps, suppositories, aerosols, cataplasmas, liniments, lotions, enemas and emulsifiers.

5

According to this invention, well-known excipients in the form of solid or liquid may be used. As mentioned in the above, the formulation should be conducted so as to contain the active ingredient of this invention necessary for single 10 dose. The several examples of excipients used in related dosage forms are as follows:

- Excipients in powders and other oral powders: lactose, crystalline cellulose, starch, dextrin, calcium phosphate, calcium carbonate, synthetic and natural aluminum dioxide, 15 magnesium oxide, dried aluminum hydroxide, magnesium stearate, and sodium bicarbonate;

- Excipients in topical powders: zinc oxide, talc, starch, kaolin, borate powder, zinc stearate, magnesium stearate, magnesium carbonate, precipitated calcium 20 carbonate, bismuth subgallate, and potassium aluminum sulfate powder;

- Excipients in liquids: water, glycerin, propylene

glycol, sweet-taste syrup, ethanol, fatty oil, ethylene glycol, polyethylene glycol, and sorbitol;

- Excipients in ointments: hydrophobic or hydrophilic base (including oil-soluble base, water-soluble base and suspended base) prepared by mixing fat, fatty oil, lanoline, vaseline, glycerin, wax, Japan wax, paraffin, paraffin sulfate, resins, higher alcohols, plastics, glycols, water, or surfactant.

10

EXAMPLE : Preparation of ginsenoside Mc

The suspended solution of human flora was precultured in GAM medium overnight and then, 100mg of ginsenoside Rc was added to said medium to the desired concentration of 2% in a newly sterile GAM medium and then cultured at 37°C for 1 day. The medium was extracted by 1-butanol and the extracted solution was concentrated and purified on reversed/irreverese phase chromatography to give 25mg of pure ginsenoside Mc.

20 [Formulation example]

- Formulation example 1 : A mixture of lactose, crystalline cellulose and 1% magnesium stearate was added to 30mg of

compound K, an intestinal flora metabolite of ginseng saponin, for homogenous mixing. Said mixture was tabletted by a tabletting machine to obtain each tablet containing 200mg.

• Formulation examples 2~4 : Based upon the same procedure 5 as described in formulation 1, each preparation was obtained containing 30mg of compound Y, ginsenoside Mc or 20(S)-protopanaxatriol, respectively, instead of 30mg of compound K.

• Formulation example 5 : A solution of 15mg of compound K, an intestinal flora metabolite of ginseng saponin and 10 polysorbit 80 was filled into a sterilized vial aseptically and after removing the moisture, the preparation for injection was obtained.

• Formulation example 6~8 : Based upon the same procedure as described in formulation 5, each preparation was obtained 15 containing 15mg of compound Y, ginsenoside Mc or 20(S)-protopanaxatriol, respectively, instead of 15mg of compound K.

The physiological actions involved in the intestinal flora metabolite of ginseng saponin of this invention are described in the following examples but these protective 20 scopes are not confined to said examples.

[Experiment 1]

Antitumor activity on leukemia cell line (P388) in lymphocyte
of mice

a) Experimental method

5 Spleen lymphocytes of mice and leukemia cell line (P388)
were used for this experiment. Spleen lymphocytes (4×10^6
cells) and leukemia cells (P388) (2×10^6 cells) were
cultured in a medium (RPMI 1640 supplemented with $20 \mu\text{M}$
mercaptoethanol and 10% fetal bovine serum) containing
10 intestinal flora metabolite of ginseng saponin ($2.5 \mu\text{M}$) in
5% CO₂ saturated with steam for 16 hours.

Aside from this, same numbers of spleen lymphocytes or
leukemia cell line were cultured in a medium containing
intestinal flora metabolite of ginseng saponin in same
15 concentration as a control. The number of each survived
cell was purified by MTT method to calculate impaired rate
of cells on leukemia cells (P388) of lymphocytes.

b) Experimental results

20 As shown in table 1, the experimental results revealed
that all intestinal flora metabolites of ginseng saponin
in a low concentration of $2.5 \mu\text{M}$ exhibited antitumor

activities 1.6 to 2 times as higher as control group.

Table 1. Antitumor activity on the cancer cells of lymphocyte by intestinal flora metabolites of ginseng saponin

	Concentration($\mu\text{g}/\text{mL}$)	Antitumor activity(%)	
Control		31.3	1
Compound K	1.56	51.5	1.6
Compound Y	1.86	56.6	1.8
Ginsenoside Mc	1.86	63.3	2.0
20(S)-protopanaxatriol	1.19	82.4	2.0

10

[Experiment 2]

Inhibition on the vascularization of tumor

(Test for the inhibition on the proliferation)

15 a) Experimental method

Human lymphocyte (HL), leukemia cell line (K562) and bovine artery endotheliocyte (BAE) were used for this experiment.

HL (1×10^5 cells), leukemia cell line (K562) (2×10^5 cells) and BAE (5×10^3 cells) were cultured in a medium (HL, K562: RPMI

20 1640 medium containing 10% fetal bovine, BAE: DMEM medium containing 10% fetal bovine) containing intestinal flora metabolites of ginseng saponin concentrated with 2-fold

dilution in 5% CO₂ saturated with steam (HL, KS62: 24 hours, BAE: 72 hours). The number of each survived cell was purified by MTT method to calculate 50% inhibition concentration (IC₅₀), impaired rate of cells (IC₅₀(HL)/IC₅₀(BAE) and IC₅₀(KS62)/IC₅₀(BAE)).

5 b) Experimental results

As shown in table 2, the experimental results revealed that ginsenoside, Mc and 20(S)-protopanaxatriol exhibited 10 inhibitory activities on the proliferation of tumor cells

Table 2. Inhibitory activity on the proliferation by intestinal flora metabolites of ginseng saponin

	IC ₅₀ (μ M)			IC ₅₀ (C)/IC ₅₀ (BAE)		
	HL	KS62	BAE	C=HL	KS62	
15	Compound K	45	26	28	1.7	1.6
	Compound Y	83	78	32	2.6	2.6
	Ginsenoside Mc	220	480	26	8.5	18
	20(S)-protopanaxatriol	280	49	36	7.8	1.4

20 [Experiment 3]

Inhibition on the vascularization of tumor

(Test for the inhibition on the migration)

a) Experimental method

Bovine artery endotheliocyte (BAE) was used for this experiment. BAE (5X10³ cells) was cultured in 6-well plate for 24 hours and cells attached to the plate were detached 5 by a razor. Said medium was replaced by a new one and after one hour, a solution of intestinal flora metabolite of ginseng saponin was added to the desired inhibitory concentration of 10% and 50%, respectively for 24-hour cultivation. After completing the cultivation, cells were 10 fixed with methanol, stained with Giemsa method and counted the cells migrated from the detached line under microscope.

b) Experimental results

15 As shown in table 3, the experimental results revealed that each intestinal metabolite of ginseng saponin exhibited an inhibitory activity of migration and among them, it was noted that compound K showed more potent inhibitory activity of migration than Suramin (Wako Pure 20 Chem. Ind. Ltd., Japan), a control group.

Table 3. Migration-inhibition by intestinal flora metabolites
of ginseng saponin

	Migration-inhibition (% control)	
	IC ₁₀	IC ₅₀
S	Suramin	-3.8
	Compound K	4.6
	Compound Y	-3.7
	Ginsenoside Mc	-1.6
	20(S)-protopanaxatriol	-1.6

[Experiment 4]

10 Inhibition on the extravasation of basement membrane

a) Experimental method

The transwell culture chamber was used for this experiment with a haptoinvasion method (reference: Cancer Res., 47, 15 3239, (1987)). The lower side of a filter having a hole of 8.0 μ m in diameter was coated with 5 μ g of matrigel for the fabrication of matrigel/FN filter. Human adenosarcoma tissue cell (HT1080), treated in the intestinal flora metabolite of ginseng saponin in a concentration of 1-1000 μ M at 37°C for 30 minutes, was charged to the upper side of each filter with 1x10⁵ cells/100 μ L. Then, said filters were added to 24-well plate having 600 μ L of MEM medium

supplemented with 0.1% bovine serum albumin and them
cultured for 4-hour cultivation. After completing the
cultivation, cells were fixed with methanol and stained
with hematoxylin, a tissue staining agent. Following the
5 removal of cells at the upper side with a cotton pole,
cells infiltrating into the lower side were counted under
microscope.

b) Experimental results

10 As shown in table 4, the experimental results revealed
that each intestinal metabolite of ginseng saponin
exhibited more potent inhibitory activity of
extravasation than RGDS peptide (under development from
Glycomed Co.:Cancer Res., 49, 3815 (1989)), a control group and
15 among them, 50% extravasation-inhibitory concentration of
compound K showed a potent activity in a low concentration
of $3.2\mu M$.

Table 4. Inhibition on the extravasation of basement membrane
20 by intestinal flora metabolites of ginseng saponin

	Concentration (μ M)	No. of infiltrated cancer cell/field	Inhibition rate (%)
Control		118 \pm 8	
RGDS peptide	4000	61 \pm 9	48
Compound K	1	73 \pm 4	38
	ED ₅₀ =3.2		
	10	45 \pm 9	62
	100	0	100
5			
Control		117 \pm 9	
RGDS peptide	4000	51 \pm 10	56
Compound Y	1	125 \pm 7	
	10	92 \pm 11	21
	ED ₅₀ =31		
	100	24 \pm 2	62
10			
	1000	0	100
Control		117 \pm 9	
RGDS peptide	4000	51 \pm 10	56
ginsenoside Mc	1	114 \pm 12	3
	ED ₅₀ =7.6		
	10	44 \pm 7	62
	100	3 \pm 1	97
15			
	1000	0	100
Control		97 \pm 8	
RGDS peptide	4000	49 \pm 4	49
20(S)-protopanaxatriol	1	103 \pm 14	
	10	93 \pm 7	4
	ED ₅₀ =48		
	100	18 \pm 4	81
20			
	1000	1 \pm 1	99

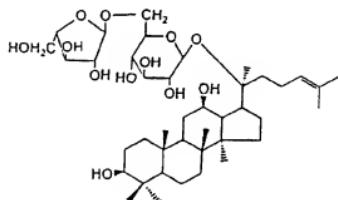
From the aforementioned results, it is noted that intestinal flora metabolites of ginseng saponin, such as compound K, compound Y, 20(S)-protopanaxatriol including ginsenoside Mc of this invention, are novel types of 5 potential anticancer agent since they have immunopotentiating actions including inhibitory actions on the vascularization of tumors and extravasation of cancer cells.

The toxicity of ginsenoside Mc, a novel compound of 10 this invention, is nearly negligible in some animal experiments with rats and mice and the products' stability of each preparation based upon each formulation example is quite effective.

WHAT IS CLAIMED IS :

1. A new compound of 20-O-[α -L-arabinofuranosyl(1 \rightarrow 6)- β -D-glucopyranosyl]-20(S)-protopanaxadiol (ginsenoside Mc) with
5 the following formula.

10



2. anticancer agent containing one active ingredient selected
from 20-O-[α -L-arabinofuranosyl(1 \rightarrow 6)- β -D-glucopyranosyl]-20(S)-
15 protopanaxadiol(ginsenoside Mc), 20-O-[β -D-glucopyranosyl]-
20(S)-protopanaxadiol(compound K), 20-O-[α -L-arabinopyranosyl(1 \rightarrow 6)- β -D-
glucopyranosyl]-20(S)-protopanaxadiol(compound Y), or
20(S)-protopanaxatriol, together with one or more
pharmaceutically acceptable carriers.

20

INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR 96/00281

A. CLASSIFICATION OF SUBJECT MATTER

IPC⁶: C 07 J 17/00; A 61 K 31/58

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC⁶: C 07 J 17/00; A 61 K 31/58

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

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QUESTEL-DARC, CAS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Chemical abstracts, Vol. 124, (Columbus, Ohio, USA), abstract No. 283 952, J.H.SUNG et al. "Metabolism of ginseng saponins by human intestinal bacteria", & Saengyak Hakhoechi, 1995, 26(4), 360-7.	1
A	Patent Abstracts of Japan, unexamined applications, Section C, Volume 7, No. 143, 22 June 1983 (22.06.83), The Patent Office Japanese Government, page 135 C 172, No. 58-57399 (OOSAKA YAKUHIN KENKYUSHO K.K.) ----	1

<input type="checkbox"/>	Further documents are listed in the continuation of Box C.	<input type="checkbox"/>	See patent family annex.
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10 June 1997 (10.06.97)	02 July 1997 (02.07.97)
Name and mailing address of the ISA/AT AUSTRIAN PATENT OFFICE Kohlmarkt 8-10 A-1014 Vienna Facsimile No. 1/53424/535	Authorized officer Hofbauer Telephone No. 1/53424/225